REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
2010	Open Literature	

4. TITLE AND SUBTITLE
A liquid chromatographic–mass spectrometric (LC–MS) method for the analysis of the bis-pyridinium oxime ICD-585 in plasma: Application in a guinea pig model

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Capacio, BR, Dusick, B, Smith, JR, McDonough, JH, Shih, T-M.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDT-D
3100 Ricketts Point Road
Aberdeen Proving Ground, MD
21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-P09-023

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDZ-I
3100 Ricketts Point Road
Aberdeen Proving Ground, MD
21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
Published in special issue of Journal of Chromatography B, 878,1420–1425, 2010. This work was supported by the Defense Threat Reduction Agency—Joint Science and Technology Office, Medical S&T Division.

14. ABSTRACT
See reprint.

15. SUBJECT TERMS
Nerve agent, bis-Pyridinium oxime(s), Pharmacokinetics, Analysis, Liquid chromatography, Mass spectrometry, GC–MS, chemical warfare agents

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Benedict R. Capacio
UNLIMITED	UNLIMITED	UNLIMITED	UNLIMITED	6	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-1944

Journal of Chromatography B, 878 (2010) 1420–1425

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A liquid chromatographic–mass spectrometric (LC–MS) method for the analysis of the bis-pyridinium oxime ICD-585 in plasma: Application in a guinea pig model☆,☆☆,★

B.R. Capacio*, B. Dusick, J.R. Smith, J.H. McDonough, T.-M. Shih

US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Rd., Aberdeen Proving Ground, MD 21010-5400, United States

ARTICLE INFO

Article history:
Received 26 August 2009
Accepted 4 December 2009
Available online 16 December 2009

Keywords:
Nerve agent
bis-Pyridinium oxime(s)
Pharmacokinetics
Analysis
Liquid chromatography
Mass spectrometry
GC–MS

ABSTRACT

In recent animal studies, several novel oxime compounds that are better than 2-PAM as antidotes against selected organophosphate (OP) nerve agents have been identified. The purpose of this study was to develop and validate a liquid chromatographic–mass spectrometric (LC–MS) method for analysis of the bis-pyridinium oxime ICD-585 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-propane) in plasma and to establish the utility of the method in a guinea pig model. Calibration curves were prepared using ICD-585-spiked plasma at concentrations from 0.156 to 10 μg/ml. Curves were run over a 1-month time frame and a total of 13 ($n = 13$) were generated. The lower limit of quantification (LLOQ) was determined to be 0.216 μg/ml. Intra- and inter-day variability was assessed by studying precision and accuracy. For intra-day studies, data from the precision determinations indicated that the % CV's ranged from 4.28 to 14.98%. The % error in the accuracy assessments ranged from −8.73 to 4.61%. For inter-day studies, precision data ranged from 3.53 to 13.20%. The % error in the accuracy assessments ranged from 0.39 to 13.77%. Room temperature, freeze–thaw and autosampler stability was also examined. For all 3 stability studies, the compound remained within ±15% of the initial analysis. Application of the method was demonstrated by analyzing samples from guinea pigs challenged with sarin (GB) or cyclosarin (GF) ($1\times LD_{50}$) followed with intramuscular ICD-585 (58 μM/kg, 21.8 mg/kg). At 55 min after oxime administration, mean (±SD) plasma concentrations were 15.98 (±4.88) μg/ml and 14.57 (±3.70) μg/ml in GB- and GF-exposed animals, respectively. In summary, studies have been carried out to verify the sensitivity, precision and accuracy of the assay as well as the stability of the analyte under various conditions. The method has been demonstrated to be applicable to the analysis of plasma from nerve agent-exposed guinea pigs.
Published by Elsevier B.V.

1. Introduction

Organophosphorus (OP) nerve agents are extremely potent inhibitors of the enzyme acetylcholinesterase (AChE). Toxicities resulting from cholinergic over-activity may result in lethality. For some nerve agents (i.e., sarin [GB], VX), enzyme inhibition can be reversed with post-exposure treatment consisting of atropine and oximes such as pralidoxime chloride (2-PAM). This process occurs by nucleophilic attack of the oxime on the phosphorus atom of the agent molecule bound to the enzyme. Subsequently, the oxime-phosphonate moiety is released along with the regenerated enzyme, which is then capable of normal AChE function [1]. For other agents such as tabun (GA), soman (GD), cyclosarin (GF) and Russian V-agent (VR), 2-PAM is either less effective or ineffective in reversing AChE inhibition, resulting in reduced survival. Pyridostigmine bromide pretreatment has been found to significantly enhance the efficacy of 2-PAM in GD and GA intoxication, but provides no additional protection in GF and VR intoxication [2,3]. Recent studies have indicated that enzyme inhibition with these latter agents is amenable to treatment with novel broad-spectrum bis-pyridinium oximes such as ICD-585, and others (HLö7 [ICD-2445], MMB4 [ICD-039], and HI-6) [4,5,6,7]. To establish the relationship between *in vivo* concentrations and efficacy in OP intoxication, a method was developed for the analysis of these compounds from plasma. A liquid chromatographic–mass spectrometric

☆ This paper is part of the special issue 'Bioanalysis of Organophosphorus Toxicants and Corresponding Antidotes', Harald John and Horst Thiermann (Guest Editors).
☆☆ Presented at the 12th Medical Chemical Defence Conference, 22–23 April 2009, Munich, Germany.
★ *Disclaimer:* The opinions, interpretations, conclusions, and recommendations are those of the author(s) and are not necessarily endorsed by the US Army. This research complied with the Animal Welfare Act and implementing Animal Welfare Regulations and adhered to the principles noted in *The Guide for the Care and Use of Laboratory Animals*.
* Corresponding author. Tel.: +1 410 436 1944.
E-mail address: benedict.capacio@us.army.mil (B.R. Capacio).

1570-0232/$ – see front matter Published by Elsevier B.V.
doi:10.1016/j.jchromb.2009.12.011

(LC–MS) method for the oxime ICD-585 is specifically addressed herein.

2. Materials and methods

2.1. Chemicals/blood/plasma

Guinea pig blood was obtained from animals involved in US Army Medical Research Institute of Chemical Defense (USAMRICD) Institute Animal Care and Use Committee (IACUC) approved protocols. Animals were maintained under an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) program. Plasma from swine (*Sus scrofa*) was purchased from Archer Farms, Inc. (Darlington, MD). Hartley guinea pig plasma derived from sodium heparin anticoagulated whole blood was obtained from Bioreclamation Inc. (Hicksville, NY). Acetonitrile and ammonium acetate were obtained from Fischer Chemicals (Fair Lawn, NJ) and used without purification. Formic acid was obtained from Matheson, Coleman, and Bell (East Rutherford, NJ). Water used was purified using a Purelab Ultra Analytic water purifier from Elga (High Wycombe, Bucks, UK). HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane) and ICD-585 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-propane), both dichloride salts, were obtained from the Walter Reed Army Institute of Research (WRAIR) (Silver Spring, MD).

2.2. Instrumentation

2.2.1. Liquid chromatography (LC)

Chromatographic separation of the samples was achieved using a Luna silica LC column, 5 μm, 250 mm × 2.0 mm (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile/water (15/85, v/v), 1 mM ammonium acetate, and 0.1% formic acid. The flow rate was 0.2 ml/min delivered isocratically and the injection volume was 10 μl. The choice of HI-6 as an internal standard was based upon its structural similarity with the analyte of interest (ICD-585). Therefore, the two compounds were expected to display similar behavior in the sample extraction and the LC separation processes. Following LC separation, the solvent stream was passed through an ultraviolet diode array detector and then directed into a mass spectrometer. Ultraviolet absorbance was measured at 254 and 302 nm using a bandwidth of 4 nm with no reference wavelengths used. A blank consisting of the acetonitrile/water in the same ratio as the mobile phase was run following each spiked plasma sample.

2.2.2. Mass spectrometry (MS)

Samples were introduced into a Hewlett Packard G1946A quadrupole mass spectrometer via an atmospheric pressure electrospray ionization (ESI) interface. The following MS conditions were used: positive ion selected ion monitoring of m/z 285 and 257 for ICD-585 and HI-6 (internal standard), respectively; fragmentor at 60 V; and capillary voltage at 4000 V. The nitrogen drying gas was introduced at a flow rate of 13 L/min and kept at 350 °C. Nitrogen was also used as the nebulization gas and maintained at a pressure of 35 psi.

2.3. Procedures

2.3.1. Sample preparation

Swine plasma was commercially purchased and utilized in the preparation of calibration curves and all method validation studies (precision and accuracy, room temperature stability, repeated freeze–thaw, and autosampler stability). Whole blood obtained from guinea pig test animals was centrifuged at 2000 × *g* for 30 min at 5 °C and the plasma supernatant removed for analysis. Commercially purchased guinea pig plasma was used to construct calibration curves, which were analyzed along with all plasma from the test animals for quantification. The sample preparation procedure was as follows. A 100-μl aliquot of plasma was transferred to a plastic microfuge vial, and 20 μl (100 μg/ml) of the internal standard (IS) HI-6 was added (final concentration 16.6 μg/ml). Following addition of acetonitrile (100 μl), the mixture was vortexed and centrifuged at 4300 × *g* for 15 min. The supernatant was removed, placed in a new microfuge vial, and centrifuged for an additional 15 min. A portion of the supernatant (150 μl) was removed, and 100 μl of the aqueous component of the mobile phase (1 mM ammonium acetate, and 0.1% formic acid) was added. The sample was then vortexed and centrifuged at 4300 × *g* for 15 min, following which it was transferred to a limited volume autosampler vial for analysis. A blank consisting of the acetonitrile/water in the same ratio as the mobile phase was run following each spiked plasma sample.

2.3.2. Calibration curves

Calibration curves utilizing swine plasma were used for method validation studies. For quantifying ICD-585 in guinea pig plasma samples, calibration curves were prepared in commercially purchased guinea pig plasma and analyzed along with the samples. All calibration curves were prepared by spiking plasma with ICD-585 to yield a concentration of 10 μg/ml. This sample was serially diluted with clean (blank) plasma. The final calculated concentration levels used for the calibration curves were 0.16, 0.31, 0.63, 1.25, 2.50, 5.00 and 10.00 μg/ml. For the method validation studies, calibration curves were prepared and each concentration level was analyzed in triplicate daily over a 1-month time frame. A total of 13 (n = 13) curves were generated. The area under the curve (AUC) ratio (analyte/internal standard) at each concentration was determined, and the concentration calculated based on the respective daily curve. Across days, mean concentration, coefficient of variability expressed as a percentage (% CV) and percent error (% error) were calculated at each concentration level. The % CV was calculated by (standard deviation [SD]/mean) × 100%. The % error was calculated by ([calculated concentration − actual concentration]/actual concentration) × 100. The limit of detection (LOD) and lower limit of quantification (LLOQ) were determined by the relationship $3S_0$ and $10S_0$, respectively [8,9]. The S_0 was calculated and derived from the y intercept of plots of standard deviations from calibration curves as a function of the 5 lowest ICD-585 concentrations, respectively [8].

2.3.3. Precision and accuracy

Intra- and inter-day variability was used to assess precision and accuracy. For intra-day studies, the assessment of precision and accuracy was carried out for assays conducted within a single day. For those studies, 6 sets of plasma test samples (n = 6) were prepared with oxime ICD-585 at 4 concentration levels (0.94, 1.88, 3.75 and 7.50 μg/ml) and analyzed the same day following the sample preparation procedure. For sample quantification, the AUC ratios (analyte/internal standard) were used in the regression equation generated from the mean data of 6 previously analyzed calibration curves. A continuing calibration verification (CCV) standard (10 μg/ml) was analyzed prior to and after the plasma test samples. Precision was assessed by calculating the SD and the % CV at each plasma concentration level. Accuracy was expressed as % error by examining the difference between the expected and mean calculated concentration of the sample. For inter-day studies, plasma test samples were prepared daily for 5 days (n = 4) using 5 concentration levels of ICD-585 (0.47, 0.94, 1.88, 3.75 and 7.50 μg/ml). Precision and accuracy were determined as described in the intra-day studies. The AUC ratios (analyte/internal standard) were used in the

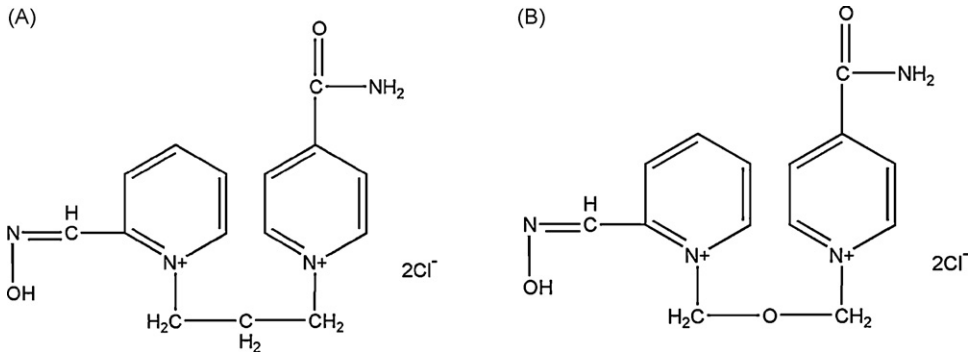

Fig. 1. Chemical structure of ICD-585 (A) and HI-6 (B).

regression equation generated from the mean data of 6 previously analyzed calibration curves. A CCV (10 μg/ml) was also included for analysis each day.

2.3.4. Room temperature stability studies

The room temperature stability was examined by preparing plasma samples containing 0.63, 1.25, 2.50, 5.00 and 10.00 μg/ml of ICD-585. Three aliquots ($n = 3$) from each sample were prepared and analyzed immediately. The samples were stored at room temperature followed by repeated preparation and analysis of 3 aliquots at each concentration level at 6 and 24 h. All data from these experiments were expressed as AUC ratios at each concentration. Data from the 6- and 24-h studies were compared with the initial analysis by calculating the percent of the initial analysis.

2.3.5. Repeated freeze–thaw study

An experiment to assess the effect of repeated freeze–thaw cycles on analyte stability was carried out by examining plasma samples at 5 concentration levels of ICD-585 (0.63, 1.25, 2.50, 5.00 and 10.00 μg/ml). Initially, 3 ($n = 3$) aliquots from each concentration level were prepared and analyzed immediately. The plasma samples were then stored at −70 °C, and for the next 4 consecutive days, they were thawed, and 3 aliquots from each concentration were removed, prepared and analyzed; the remainder was refrozen to await the next analysis. Samples underwent a total of 4 consecutive freeze–thaw cycles subsequent to the initial analysis. All data from these experiments were expressed as area under the curve (AUC) ratios (analyte/internal standard) at each concentration. Data from each of the freeze–thaw cycles were compared with the initial analysis by calculating the percent of the initial analysis.

2.3.6. Autosampler stability study

The stability of prepared plasma samples while on the autosampler was examined. Plasma samples containing 0.16, 0.31, 0.63, 1.25, 2.50, 5.00 and 10.00 μg/ml of ICD-585 were prepared as described in Section 2.3.1. The samples were placed in the autosampler and immediately analyzed. The prepared samples were allowed to sit on the autosampler and re-analyzed after 3 and 6 h. This study was carried out on 6 ($n = 6$) and 4 ($n = 4$) separate days for the 3- and 6-h studies, respectively. All data from these experiments were expressed as AUC ratios at each concentration. Data from the 3- and 6-h studies were compared with the initial analysis by calculating the percent of the initial analysis.

2.3.7. Application of the method

Whole blood was obtained from guinea pigs ($n = 8$) treated with intramuscular (im) ICD-585 (58 μM/kg, 21.8 mg/kg) 5 min after 1× LD_{50} GF or GB. The blood was obtained at 55 min following oxime administration. The blood was anticoagulated with heparin and centrifuged for 30 min (2000 × *g* at 5 °C). The plasma was removed from the red blood cell fraction and stored at −70 °C until use.

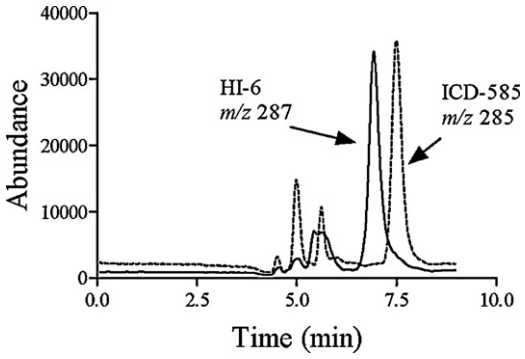

Fig. 2. Reconstructed ion-chromatogram: a representative reconstructed ion-chromatogram of ICD-585 (0.625 μg/ml) with internal standard (HI-6) following work-up from plasma. Ions monitored were $m/z = 285$ and $m/z = 287$ for ICD-585 and HI-6, respectively.

3. Results

3.1. Calibration curve study

The chemical structures of ICD-585 and HI-6 are shown in Fig. 1. A representative mass-chromatogram from the standard curve depicting ICD-585-spiked plasma (0.63 μg/ml) prepared as described (with HI-6 as IS) is shown in Fig. 2. The mass-chromatogram demonstrated that the peaks of interest were baseline resolved and free of any interference arising from the plasma matrix. Calibration curves were prepared using ICD-585-

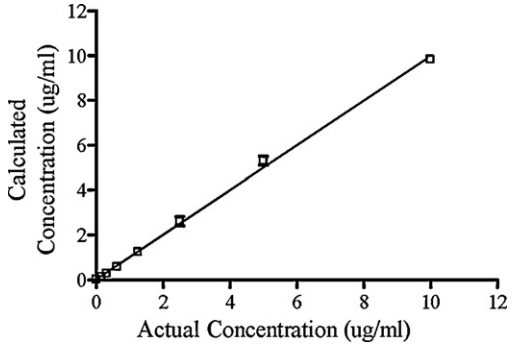

Fig. 3. Calibration curves for ICD-585-spiked plasma were prepared and each concentration level analyzed in triplicate over a 1-month time frame. A total of 13 ($n = 13$) curves were generated. Data represent mean calculated concentrations ±SD as a function of actual concentration ($R^2 = 0.9983$).

Table 1
Calibration curves for ICD-585-spiked plasma were prepared and analyzed in triplicate over a 1-month time frame. Data at each concentration represent mean calculated concentration ($n = 13$): SD = standard deviation; % CV = percent coefficient of variation (SD/mean × 100); % error = ([calculated concentration − actual concentration]/actual concentration) × 100.

Actual concentration (μg/ml)	Mean calculated concentration (μg/ml)	Precision % CV	Accuracy % error
0.16	0.12	43.37	−22.26
0.31	0.27	14.74	−14.63
0.63	0.57	8.61	−8.08
1.25	1.24	8.56	−0.81
2.50	2.62	7.84	4.74
5.00	5.31	3.75	6.11
10.00	9.82	1.07	−1.76

spiked pig plasma at concentrations from 0.16 to 10 μg/ml. The AUC ratios (analyte/internal standard) at each concentration were determined, and the concentration was calculated based on the respective daily curve. The calculated concentrations were plotted as a function of theoretical concentration. Calibration curves were run over a 1-month time frame and a total of 13 ($n = 13$) curves were generated. Correlation coefficients (R^2) for each curve ranged from 0.9928 to 0.9995. A plot of the mean ($n = 13$) calculated concentration (±SD) as a function of actual concentration ($R^2 = 0.9982$) is presented in Fig. 3. The % CV's derived from the mean AUC ratios ranged from 43.37 to 1.07%; the % errors ranged from −22.26 to 6.11% (Table 1). The greatest % CV (43.37%) and % error (−22.26%) were found at the lowest concentration level (0.156 μg/ml); all others were less than 15%. The LOD ($3S_0$) and LLOQ ($10S_0$) were calculated as 0.065 and 0.216 μg/ml, respectively.

3.2. Precision and accuracy

3.2.1. Intra-day variability

Precision and accuracy data for intra-day plasma test samples are presented in Table 2. Data from the precision determinations indicated that the % CV's ranged from 4.28 to 14.98%. The % error in the accuracy assessments ranged from −8.73 to 4.61%. The % CV and % error data for the 10 μg/ml CCV were 4.07 and −0.63%, respectively.

3.2.2. Inter-day variability

Precision and accuracy data for inter-day plasma test samples are presented in Table 3. Precision data ranged from 3.53 to 13.20%. The % error in the accuracy assessments ranged from 0.39 to 13.77%. The % CV and % error data for the 10 μg/ml CCV were 3.85 and 4.53%, respectively.

Table 2
Intra-day variability study: six sets of plasma test samples ($n = 6$) were prepared at 4 oxime concentration levels in a single day. The samples were analyzed immediately following the work-up procedure. Precision was assessed by calculating the percentage coefficient of variation (% CV) at each plasma concentration level. Accuracy was expressed as percentage error (% error) by examining the difference between the expected and mean calculated concentration of the sample [((calculated − expected)/expected) × 100]. Continuing calibration verification (CCV) standards (10 μg/ml) were analyzed prior to ($n = 2$) and after ($n = 2$) the plasma test samples. The concentrations of the oxime were calculated based on the regression equation generated from the mean data of 6 previously analyzed calibration curves.

Actual concentration (μg/ml)	Mean calculated concentration (μg/ml) ($n = 6$)	Precision % CV	Accuracy % error
0.94	0.85	14.98	−8.73
1.88	1.94	11.50	3.51
3.75	3.92	7.22	4.61
7.50	7.16	4.28	−4.57
10.00 (CCV) ($n = 4$)	9.94	4.07	−0.63

Table 3
Inter-day variability study: plasma test samples were prepared daily for 4 days ($n = 4$) using the same concentration levels as those for the intra-day studies. Samples were analyzed each day immediately following preparation. Continuing calibration verification (CCV) standards (10 μg/ml) were analyzed daily. Precision and accuracy were determined as described in the intra-day studies. The concentrations of the oxime were calculated based on the regression equation generated from the mean data of six previously analyzed calibration curves.

Actual concentration (μg/ml)	Mean calculated concentration (μg/ml) ($n = 4$)	Precision % CV	Accuracy % error
0.47	0.51	12.08	8.34
0.94	1.02	13.20	8.53
1.88	2.13	7.02	13.77
3.75	4.21	5.93	12.38
7.50	7.53	3.53	0.39
10.00 (CCV) ($n = 4$)	10.45	3.85	4.53

3.3. Room temperature stability study

Room temperature stability of ICD-585 in plasma was examined by analyzing unprocessed samples stored on the bench top for 6 and 24 h. The data from this study indicate that room temperature storage of plasma samples for up to 24 h did not impact sample integrity (Fig. 4). Curves in Fig. 4 depict the mean values ($n = 3$) for initial (no storage; square), 6-h (triangle), and 24-h (circle) storage data along with calculated curves corresponding to ±15% (solid black lines) of the initial analysis. The plasma concentrations of ICD-585 tested (0.63–10 μg/ml) after room temperature storage were at most ±9.3% at 6 h and ±6.6% at 24 h of the initial analysis values (Fig. 4). The R^2 values ranged from 0.9982 to 0.9996.

3.4. Repeated freeze–thaw study

An experiment to assess the effect of repeated freeze–thaw cycles on analyte stability in plasma was carried out. The data indicate that repeated freeze thawing through 4 cycles did not impact the integrity of the samples (Fig. 5). Curves in Fig. 5 demonstrate the mean values ($n = 3$) for the initial analysis (no freezing; square), along with data ($n = 3$) from each of the freeze–thaw cycles (1–4). Also shown are calculated curves corresponding to ±15% (solid black lines) of the initial analysis. The plasma concentrations of ICD-585 tested (0.625–10 μg/ml) were ±12.5% of the initial analysis values with the majority of the samples falling within ±6% (Fig. 5). The R^2 values ranged from 0.9958 to 0.9991.

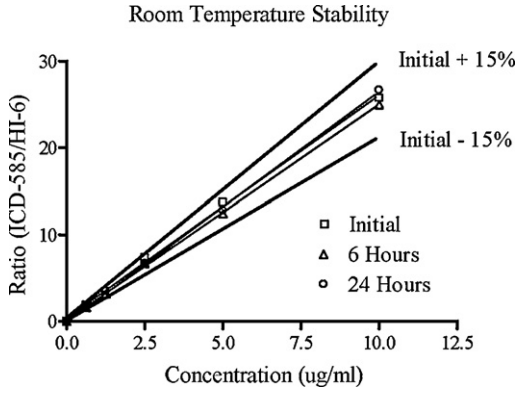

Fig. 4. Room temperature stability of ICD-585 was examined by processing and analyzing plasma samples that were stored on the bench top for 6 and 24 h. The curves depict the mean values ($n = 3$) for the initial (no storage; square), 6- (triangle), and 24-h (circle) storage data along with calculated curves corresponding to ±15% (solid black lines) of the initial analysis.

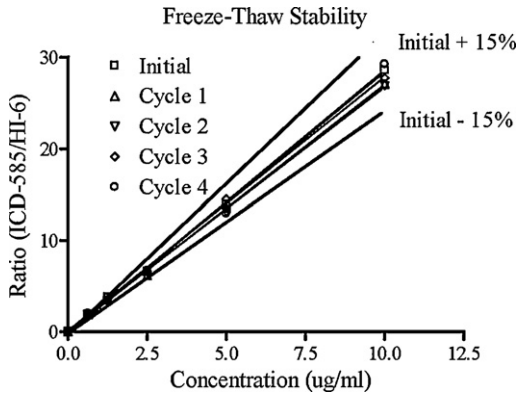

Fig. 5. Freeze–thaw stability: 3 plasma samples from each concentration were analyzed immediately following the work-up procedure. Samples were then stored at −70 °C, and for the next 4 consecutive days, they were thawed, and 3 aliquots from each concentration were removed, prepared and analyzed; the remainder was refrozen to await the next analysis. The curves depict the mean values ($n = 3$) for the initial analysis, along with data ($n = 3$) from each of the freeze–thaw cycles (1–4). Also shown are calculated curves corresponding to ±15% (solid black lines) of the initial analysis.

3.5. Autosampler stability study

The stability of ICD-585 in processed plasma samples while stored in the autosampler was studied by analyzing them immediately, 3 and 6 h following preparation each day. The data from these experiments suggest that the prepared samples remained intact for up to 6 h following preparation (Fig. 6). Fig. 6 demonstrates mean data from the initial analysis (square, $n = 6$), 3 h (triangle, $n = 6$) and 6 h (circle, $n = 4$). Also shown are calculated curves corresponding to ±15% (solid black lines) of the initial analysis. The results from the studies indicate that the samples analyzed subsequent to the initial analysis were ±6.9 and ±12.5% of the initial concentrations for the 3- and 6-h analysis times, respectively (Fig. 6). The R^2 values ranged from 0.9982 to 0.9992.

3.6. Application of the method

The LC–MS method was applied to plasma obtained from guinea pigs ($n = 8$) treated with ICD-585 (im) 5 min following challenge with 1× LD_{50} of GB or GF. Whole blood was obtained at 55 min after oxime administration. Mean (±SD) concentrations of ICD-585 in plasma were 15.98 ± 4.88 (range 10.63–23.69 μg/ml) and 14.57 ± 3.7 μg/ml (range 11.22–22.12 μg/ml) in GB- and GF-challenged animals, respectively (Table 4).

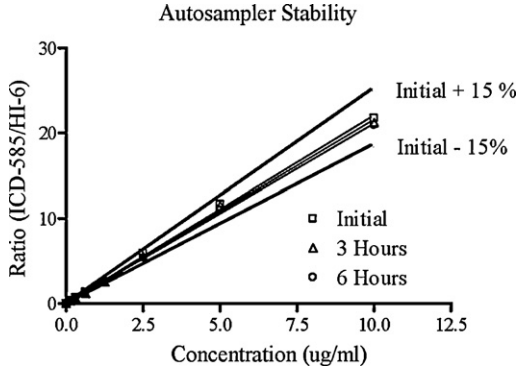

Fig. 6. Autosampler stability: plasma samples were prepared and placed in the autosampler. The prepared samples were analyzed immediately ($n = 6$, square) and again at 3 ($n = 6$, triangle) and 6 h ($n = 4$, circle). Also shown are calculated curves corresponding to ±15% (solid black lines) of the initial analysis.

Table 4
Guinea pig study. Guinea pigs ($n = 8$) were challenged with 1× LD50 GB or GF, followed by intramuscular ICD-585 (58 μM/kg, 21.8 mg/kg). Whole blood was obtained at 55 min after oxime administration and analyzed in duplicate (a and b) as described.

Animal ID	Concentration (μg/ml)	Mean
Challenge agent GB		
1a	10.58	
1b	11.47	11.03
2a	22.72	
2b	22.59	22.66
3a	14.94	
3b	14.90	14.92
4a	10.55	
4b	10.72	10.63
5a	16.57	
5b	17.02	16.79
6a	23.16	
6b	24.22	23.69
7a	14.99	
7b	14.20	14.59
8a	13.25	
8b	13.85	13.55
Mean = 15.98 μg/ml, SD = 4.88 μg/ml, % CV = 30.53		
Challenge agent GF		
1a	16.25	
1b	15.47	15.86
2a	13.25	
2b	13.59	13.42
3a	11.27	
3b	11.76	11.52
4a	11.33	
4b	11.11	11.22
5a	14.40	
5b	14.40	14.40
6a	16.58	
6b	16.72	16.65
7a	11.76	
7b	10.92	11.34
8a	21.95	
8b	22.29	22.12
Mean = 14.57 μg/ml, SD = 3.70 μg/ml, % CV = 25.33		

4. Discussion

An LC–MS method for determining the concentration of oxime ICD-585 in plasma has been developed. Relative to LC–ultraviolet analyses, LC–MS offers several advantages. Misidentification of compounds that may be similar enough chemically to co-elute in the liquid chromatographic separation is a potential problem with ultraviolet analyses. However, specificity of MS as a detection technique enables unambiguous identification of the compounds of interest even if analyte co-elution is an issue. One of the more significant advantages of MS analyses in general is a decreased requirement for a rigorous sample preparation procedure. This factor impacts on time and minimizes introduction of error from increased sample handling. Although MS offers enhanced sensitivity versus ultraviolet detection, concentrations encountered in these studies are well within the working range of ultraviolet systems. Studies have been carried out to verify the reproducibility of the assay as well as the stability of the analyte when subjected to various conditions expected during routine sample storage, preparation, and analysis.

Calibration curve analyses demonstrate that the assay was linear over the concentration range studied (0.156–10 μg/ml). The inter-day variability (i.e., over a 1-month time period) was characterized by the % CV's (1.07–43.37%) and % errors (−22.26 to 6.11%). The greatest % CV (43.37%) and % error (−22.26%) were found at the lowest concentration level examined (0.156 μg/ml), which

was less than the calculated LLOQ (0.216 μg/ml). The % CV's and % errors at all other concentrations were less than 15%. The concentration range for the calibration curves was chosen based on preliminary animal data suggesting that a human equivalent dose (58 μM/kg, 21.8 mg/kg) given im to guinea pigs would result in similar plasma concentrations. Initial data from animal experiments indicated that the range of concentrations achieved at 55 min following oxime dosing was greater than the highest concentration utilized for the standard curve. Therefore, during animal sample work-up, the initial aliquot of plasma was diluted such that concentrations calculated were within the range of the standard curve. The dilution factor was then applied to obtain the actual final concentration reported. Future studies involving complete pharmacokinetic plasma concentration–time profiles will require a wider range of concentrations on the standard curve to accommodate all concentrations encountered.

Although the calibration curve experiments demonstrated inter-day precision and accuracy, an additional study to examine intra- and inter-day variability was carried out. The study utilized different concentration levels than those employed in the calibration curves. The inter-day results support the findings from the calibration curve study in that above the LLOQ the % CV and % errors were less than 15%. In addition, the intra-day studies indicate similar results. Taken together, the data demonstrate that above the calculated LLOQ, the assay performs at levels defined as acceptable by the FDA (In: Guidance for Industry) (i.e., less than 15%) for both intra- and inter-day analysis paradigms.

Studies were conducted to examine the room temperature stability of ICD-585 in plasma. Assay of the plasma samples that were allowed to remain at room temperature for 6 and 24 h after spiking produced values that varied by less than ±10% of the initial analysis. The data indicate that plasma samples can be left on the bench top (up to 24 h) during the course of a working day prior to being processed without a compromise in integrity. The time frame is much greater than that expected during routine sample handling and preparation.

The freeze–thaw study was conducted to examine the effect of repeated freezing and thawing on analyte stability. Results from the experiments demonstrate that plasma concentrations were ±12.5% of the initial analysis values following 4 freeze–thaw cycles. The practical application of this information is that subsequent to animal experiments and the collection of blood/plasma, routine sample handling may involve initial freezing of plasma until preparation. In addition, sample analyses may need to be repeated requiring refreezing and thawing to confirm initial results if necessary. These data indicate that plasma samples can undergo at least 4 freeze–thaw cycles with no change in concentration of ICD-585 relative to that determined from the original analysis.

The stability of prepared samples awaiting injection while stored in the autosampler tray was examined. The relevance is that the proximity of the autosampler to the instrumentation can create elevated temperatures and potentially accelerate sample degradation. The results from the studies indicate that prepared plasma samples varied at most ±12.5% of the initial analysis values. All the data from the 3-h study were less than 7.0% of the initial assay; data from the 6-h study were less than 9.5% except for 0.32 μg/ml (12.5%) and 0.63 μg/kg (10.7%). These experiments indicate that relatively large numbers of samples can be prepared and placed in the autosampler for up to 6 h while awaiting analysis.

The stability study results indicate that samples will remain stable when handled within the limits of the described studies. In addition, the precision and accuracy determinations characterize the assay of ICD-585 concentrations along with associated variability as demonstrated in Tables 2 and 3.

Application of the method was demonstrated by analyzing samples from guinea pigs challenged with GB or GF 1× LD_{50} followed with im ICD-585 (58 μM/kg, 21.8 mg/kg). The blood was obtained 55 min after oxime administration. Results indicate that most of the data fall into the 11–17 μg/ml range (Table 4). However, in 3 animals (ID #2 and #6 with GB and ID #8 with GF challenge), plasma concentrations were 22–24 μg/ml (Table 4). Pharmacokinetic studies of similar bis-pyridinium oximes, MMB-4 and HI-6, have been carried out in swine (Stemler et al., 1991) [10] and sheep (Moore et al., 1988) [11]. Data from the MMB-4 studies in swine indicate that the mean maximal plasma concentration (C_{max}, 78 μg/ml) was achieved in an average of 9.1 min following im administration of 32.9 mg/kg. In sheep, the C_{max} of 20 μg/ml was observed at 13.6 min following im administration of 12 mg/kg. Taken together with the guinea pig data shown herein, and given the similarity of the compounds and route of administration, the C_{max} in guinea pigs following 21.8 mg/kg of ICD-585 would be predicted to be greater than 20 μg/ml and occur at less than 55 min. These preliminary studies were not designed to determine the peak plasma levels, but to demonstrate the feasibility and reproducibility of the assay following im administration. However, along with data from the literature, the studies provide insight as to concentration levels that may be expected in future guinea pig studies.

In summary, an LC–MS method for determining the oxime ICD-585 in plasma has been developed. Since the method utilized a structurally related analog (HI-6) as an internal standard, the same analysis can potentially be used for quantification of HI-6 with ICD-585 as the internal standard. This method offers the benefits of MS analysis (sensitivity and selectivity) along with a simple and rapid sample work-up procedure. Studies have been carried out to verify the reproducibility of the assay as well as the stability of the analyte under various conditions. The method will be the basis for developing assays for other structurally related oxime compounds. Application of the method will involve relating plasma concentrations to pharmacodynamic effects and efficacy in animals exposed to nerve agents.

Acknowledgements

This work was supported by the Defense Threat Reduction Agency—Joint Science and Technology Office, Medical S&T Division.

References

[1] J. Clement, Arch. Toxicol. 66 (1992) 143.
[2] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), 2001.
[3] J. Kassa, J. Toxicol. Clin. Toxicol. 40 (2002) 803.
[4] J. Kassa, J. Cabal, Pharmacol. Toxicol. 84 (1999) 41.
[5] I. Koplovitz, S.M. Schulz, J.R. Stewart. USAMRICD-TR-96-01, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, 1996, AD A311755.
[6] P.M. Lundy, A.S. Hansen, B.T. Hand, C.A. Boulet, Toxicology 72 (1992) 99.
[7] D.M. Maxwell, K.M. Brecht, I. Koplovitz, J. Am. Coll. Toxicol. 15 (S2) (1997) S78.
[8] D.H. Moore, F.S. Tucker, I.J. Hayward, B.J. Lukey, USAMRICD-TR-88-04. US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, 1988, AD A195609.
[9] F.W. Stemler, T.M. Tezak-Reid, M.P. McClusky, A. Kaminskis, A.K.D. Corcoran, M.L. Shih, J.R. Stewart, J.V. Wade, I.J. Hayward, Fundam. Appl. Toxicol. 16 (1991) 548.
[10] J.K. Taylor, Quality Assurance of Chemical Measurements, Lewis Publishers, Inc., Chelsea, MI, 1990, pp. 78–80.
[11] P. Taylor, in: J.H. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon, A. Goodman Gilman (Eds.), Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill, New York, 1996, pp. 161–176.